

STEN STÅHL

VARIANTS OF *MYXOCOCCUS VIRESCENS*

Characterization, growth and slime production.

Lund 1973

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Av

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INTRODUCTION

This paper is a summary of the following publications:

- I. Haskå, G. & Ståhl, S. 1971. Variants of *Myxococcus virescens* exhibiting dispersed growth. Growth and production of extracellular enzymes and slime. — *Physiol.Plant.* 24:136—142.
- II. Ståhl, S. 1972. Growth of variants of *Myxococcus virescens*. — *Ibid.*, 26:338—345.
- III. Ståhl, S. 1973. Slime of *Myxococcus virescens*. — *Ibid.* 28:523—529.

In the text below, these papers are referred to by the Roman numerals I—III.

Nature of Myxococcus virescens. The myxobacterium *M. virescens* may obtain its required nutrients by preying on other microorganisms, which are lysed by myxobacterial enzymes. In the laboratory it can be grown in several media based on hydrolyzed proteins. Depending on the environmental conditions, the myxobacterial cells will either grow vegetatively, dividing by binary fission, or enter a developmental phase terminating in the formation of a fruiting body. The swarming cells depend on a solid surface to exhibit their gliding motility, the mechanism of which is largely unknown. The morphogenetic cycle is entered when the cells move towards centres of aggregation, where fruiting bodies are subsequently formed. In connexion with the formation of the fruiting body or as a separate event, the individual rod-shaped cells will undergo morphogenesis and become spherical, refractile, resting units = myxospores. *M.virescens* is closely related, but different in pigmentation, to *Myxococcus xanthus*, which is used by many American researchers.

Over the last decade much has been learned about myxobacteria in general. Dworkin (1972) has suggested that the study of these organisms represents an unique opportunity to use procaryotes to investigate intercellular cooperation. Their ecological role might also be extensive i.e. in regulating natural microbial populations. *Statement of problems and objectives:* In liquid media, the wild types of most myxobacteria grow as aggregates. Consequently, growth can not be followed either turbidimetrically or by viable count methods. Such experimental difficulties, encountered during preliminary investigations, emphasized the need for strains of *M.virescens* with the ability to grow homogeneously dispersed in liquid media. The existence of such strains in, for example, *M.xanthus* was known from the literature (Dworkin 1966). The first objective of this investigation was therefore the isolation of similar strains in *M.virescens* (I, II).

Using strains growing in dispersion, modern microbiological methods could be employed and more accurate growth experiments, for example, became possible. It was early recognized that the results of experiments with these laboratory strains must be interpreted and generalized restrictively, until the reason for the behaviour of the organisms was known. The second objective was to compare the obtained laboratory strains with each other and the wild type (II). Although a complete characterization has not been attempted, representatives for the two major classes of strains have been used in all types of experiments.

A third objective was to investigate to what extent differences in the slime or cell envelope of cells from the two classes could explain their behaviour. Few attempts to characterize the slime components of myxobacteria (meaning slimebacteria) have been made and only one report deals with the slime of *M.xanthus* (Holt and Powelson 1960). Recently, an article dealing with the lipopolysaccharides of fruiting and non-fruiting myxobacteria has appeared (Sutherland and Smith 1973). The results so far in a biochemical and physiological investigation of the *Myxococcus virescens* slime is described in III.

Since the presented papers to some extent overlap in treating the same subjects, such as slime production and growth, the results will be presented not in chronological order but under the headings of the different subjects.

The number of references given has been kept at a minimum; complete lists will be found in the relevant papers.

RESULTS

Isolation of strains (II): All variants originated from strain B2 of *Myxococcus virescens* (Norén and Raper 1962). This wild type strain grows as aggregates in most liquid media. Since it showed this mode of growth in liquid casitone or NIII C medium, whether shaken or not, the strain was assigned the phenotype D^- (no dispersed growth) under these conditions. Consequently, the phenotype D^+ stands for growth in dispersion under comparable conditions.

Strains growing in dispersion were isolated from turbid cultures of strain B2. Several successive passages in liquid medium of cells, originating from plates kept wet during incubation, were required before such turbid cultures developed. When cells from these cultures were plated on casitone agar, two main types of colonies could be found. Cells from a "smooth" (S) colony showed dispersed growth, while cells from a "rough" (R) colony grew in films or as clumps. It was considered probable that the wild type B2 contained cells able to show both phenotypes, i.e. D^+ and D^- . Small numbers of D^+ -cells in a population dominated by D^- will probably be trapped in clumps, and therefore the equilibrium must be strongly shifted in favour of D^+ -cells for turbid cultures to develop. Strain B2 had been lyophilized and rehydrated prior to use. Since pigmentation variants could be selected after such a process it was believed possible that segregation D^- to D^+ also could be enhanced by lyophilization.

Characterization of strains (II): New variants with respect to colony morphology, pigmentation, enzyme production, etc., were obtained by further cloning and selection. They could all be assigned to one of the two major classes with respect to their mode of growth in liquid media, but in addition differences between different strains within the classes were noted. The pigmentation of the colonies varied independently of the ability to grow in dispersion. Although the best enzyme producer was a D^- -strain (S1H), strains D4a and D4b (both D^+) differed in their ability to produce bacteriolytic but not proteolytic enzymes in liquid NIII C medium. In casitone medium, however, they produced the same amount (I). All strains could be induced to form myxospores, and the D^+ -strains did not seem to be morphogenetic mutants at this stage.

The distinguishing features of the strains appeared instead to be connected with the slime or cell envelope, since phenotypes associated with D^+ were S (smooth colony), FB^- (no fruiting bodies on several media) and with D^- : R (rough colony) and FB^+ (fruiting bodies formed on the tested media). The slime of an S colony was loose, but tough and adhering in a R colony. A preliminary electron microscopical investigation of negatively stained cells from swarms on agar plates indicated that D^- -cells were surrounded by a fibrillar network, which was lacking in D^+ -cells. On aerobacter agar D^+ -cells were, however, surrounded by some type of slime material.

It must be emphasized that the phenotypes, as expected, were apparent only under certain experimental conditions. Strain D11 (D^+ in most media) will show the phenotype D^- if aerobacter cells are present in the liquid medium and will form fruiting bodies on living aerobacter cells. Strain S1H (D^- in most media) will gradually show the phenotype D^+ during cultivation in a fermentor with heavy aeration and agitation, while retaining its aggregation ability, as showed by transfers to more static cultures.

Growth on solid media (II). Growth experiments on solid media have been used in the early days of myxobacterial research and it is still an open question whether or not they reflect the natural growth of these organisms (Dworkin 1966). Both growth and swarming contribute to the increase in colony diameter with time. The method seemed, however, adequate for comparing the two classes of strains.

On casitone agar strain D11 (D^+) produced smaller colonies than strain S1H and the rate of spread was about one third of that shown by strain S1H. This indicated a difference in motility or growth rate, although

the estimated doubling time in liquid casitone medium seemed to be the same for the two strains. Strain S1H also spread about twice as rapidly on aerobacter agar as did strain D11. By addition of casitone to the aerobacter medium the rate of spread could be varied for both strains, interpreted to result from variations in enzyme production. It is noteworthy that aerobacter cells strongly stimulated the radial growth rate of strain D11.

Growth in liquid medium (I, II, III). Growth of strains D11, D4a and D4b as well as of other D⁺-strains was followed turbidimetrically as absorbancy at 700 nm. The wavelength was chosen to minimize errors in the readings due to the colour of the medium and the cells. Growth of strain S1H was followed by an indirect turbidimetric method (III). The turbidity of a disintegrated cell suspension after addition of trichloroacetic acid was read as absorbancy at 600 nm. In the case of strains D4a and D4b viable counts by the overlay method were also made (I). Due to the different methods for growth determination, the growth of D⁺ and D⁻ strains can only be compared to some extent. In general, the myxobacterial growth curve indicated a density-dependent growth consisting of several successive exponential growth phases with increasing doubling times of absorbancy and viable cells. A short stationary phase and a rapid death during the phase of decline seemed characteristic for all *M. virescens* strains under the employed conditions of cultivation.

In the main exponential phase, D⁺-strains and strain S1H grew with a doubling time (absorbancy) of 5.5 hours in liquid casitone medium (I, III). For strains D4a and D4b, the number of viable cells was doubled every 4 hours. For the latter strains the doubling time (absorbancy) was about 4 times longer in NIII C medium than in casitone. The maximal cell mass was also about 4 times higher in casitone than in casamino acids media. This confirmed the results of Dworkin (1962) that enzymatically hydrolyzed casein (Difco Casitone) was better suited for myxobacteria than acid hydrolyzed casein (Difco Casamino Acids). During growth in both media, the pH of the medium increased from about 7 to about 9 (I). The pigmentation of the medium also increased during growth of both types of strains (I, III).

During the exponential phase, bacteriolytic and, later, proteolytic enzymes were secreted into the medium. Both activity peaks appeared before the phase of decline and before DNA could be detected in the extra-cellular medium. Therefore, the enzymes were interpreted to be extra-cellular according to the definition given by Pollock (1962).

Excretion of slime (I, III). The slime was generally precipitated from the culture solution by the addition of 3 volumes of cold 96% ethanol, collected by centrifugation and assayed in the anthrone reaction. The amount of polysaccharides in the slime was arbitrarily expressed as glucose equivalents (Geq). Glucuronic acids, glucosamines, RNA or DNA were not expected to contribute significantly to the anthrone value.

In cultures of D⁺-strains, the anthrone reactive material reached one maximum during the early stationary growth phase in both media. The maximum value of ethanol precipitable slime was about 7 times higher in casitone medium (130 μ g Geq/ml) than in NIII C medium (18 μ g Geq/ml). In casitone medium, the amount of anthrone positive material decreased steeply during the beginning of the phase of decline, but a new maximum peak (150 μ g Geq/ml) followed at the end of this phase. The first peak was considered to be caused by extracellular slime but the second by material released from the cells during lysis, since DNA could be detected simultaneously. It was later shown (III) that the first maximum peak of slime for strain D11 was caused not only by the increase in cell mass or number of cells during growth but also by an increased excretion above a certain culture density.

Strain S1H excreted slime at a later stage during growth than strain D11. Only one slime peak appeared in the growth curves during the stationary phase (45 μ g Geq/ml) and the phase of decline. The excretion started before DNA could be detected in the medium, but the late excretion suggested that more slime was cell-bound in this strain. Some slime was considered to be released from the cells when the pH of the medium had increased as a result of myxobacterial activity.

Isolation and purification of slime from strains D11 and S1H (III). Slime preparations were obtained from different phases of growth in order to check on production and composition changes, as well as to check on contaminating components from medium or autolysed cells.

The crude ethanol precipitate was partly dissolved at about pH 5.4, reprecipitated by ethanol and then dissolved at the same pH (fraction A). Most of the undissolved remainders could then be dissolved at pH 9.0 (fraction B).

The yield of dialyzed and lyophilized material (sum of both fractions) ranged from about 120 μg to 260 μg per ml precipitated solution. The fractionation obtained indicated differences between the strains, in that more material from strain S1H than from strain D11 was recovered in the pH 9.0 fraction. Furthermore, the amount in this fraction increased for strain S1H while it decreased for strain D11 during growth. As growth proceeded, the content of anthrone positive material increased while Folin-positive material in the preparations decreased. All preparations also contained a few percent lipid but preparations from the phase of decline only contained DNA. Both A and B fractions of material from strain S1H contained proteolytic activity while only the A fractions of strain D11 did so. The presence of proteolytic enzymes in the slime could result from coprecipitation or adsorption to a slime component.

The high polymers of the slime were separated from material of low molecular weight and coprecipitated medium constituents by gel filtration on Sepharose 2B. During growth the relative amount of high polymers in the slime increased. Some high polymers (fraction A) seemed, however, to be degraded in the culture during the phase of decline. The low polymeric slime components could not be separated from material of casitone origin in this gel filtration step.

The final recovery of high polymeric anthrone positive material was about 10% and it had been purified from Folin-positive material at least 250-fold.

A comparison of corresponding preparations from strains D11 and S1H, i.e. with maximum content of anthrone positive material but no contaminating DNA, shows that D11 slime contained twice the amount of high polymers soluble at pH 5.4 as did S1H slime. In contrast, S1H slime contained four times the amount of high polymers soluble at pH 9.0.

DISCUSSION

Speculations on the role of the myxobacterial slime have largely been centered on the obvious manifestations of slime in fruiting body formation and on its hypothetical action in motility, but much work remains to be done before each component of the complex myxobacterial slime is identified with respect to its chemistry and biological role.

Sutherland and Smith (1973) were not able to demonstrate a clear difference between lipopolysaccharides from fruiting and non-fruiting myxobacteria. This is hardly surprising since they did not investigate fruiting and non-fruiting strains of the same species. Their results may also strengthen the view that any difference lies in the slime.

The results summarized in this paper are most easily interpreted by assuming the existence of an adhesive component in the myxobacterial slime (II). This component could be a polysaccharide, but many compounds other than pure polysaccharides, as well as fimbriae or similar appendages might be responsible for adhesiveness. Strains with a low production of such a component or with an overproduction of another slime component with ability to neutralize the adhesive effect of the former, might behave as the described D⁺-strains. Differences in excretion of slime may only reflect a modification of the cell envelope leading to a change in solubility of the slime layer (III).

The assumption that environmental factors influence the composition of the slime is not inconsistent with this view. D⁺-strains thus produce more soluble slime in casitone than in NIII C medium (I) and the slime seemed to be modified by products originating from aerobacter cells (II).

Dworkin (1972) has suggested that, in preserving the existence or potential for the swarm at all times, myxobacterial cells will resist the tendency towards dispersal. He expects myxobacteria to grow in clumps only when the medium is suboptimal in terms of growth rate. Such a view leads to the questionable conclusion that in order to function most efficiently, i.e. grow as a swarm, the myxobacteria need unfavourable environmental conditions. Although strain S1H grows in dispersion under optimal conditions of oxygen pressure and agitation, its aggregation ability is retained and the growth rate is the same as under more static conditions, when the phenotype D^- is expressed. It is possible that D^+ -strains require less oxygen than D^- -strains, to show the D^+ phenotype, i.e. to synthesize the slime components resulting in this phenotype, since the D^+ phenotype is shown even in static liquid cultures.

Why, then, does the D^+ -phenotype lead to smaller colonies and a lower rate of spread on solid casitone medium? The D^+ -strains can probably not be regarded as non-motile mutants (II). It is quite possible, however, that an adhesive component could facilitate the spread of the colony, enabling the cells to stick together and travel in groups. Another possibility is that the soluble slime acts as an inhibitor of growth or motility.

The slime may also play another role in myxobacterial nutrition. The secretion of enzymes is sequential in that bacteriolytic enzymes are found before proteolytic enzymes in the extracellular medium (I), interpreted to result from the requirement of the myxobacteria to dissolve the cell wall of the food organisms before the cytoplasm can be digested. Exoenzymes that freely diffuse away from the cell that made them seems uneconomical for the individual cell but perhaps not for the swarm as a whole. Some "exoenzymes" might, however, be slime-bound. If this were true, D^+ -strains could be expected to release more proteolytic activity as exoenzymes than D^- -strains, which seems valid for strain D4a with five proteases as exoenzymes in contrast to strain S1H with one or two at most (Gnosspelius 1972, Fil.lic. Thesis). This interpretation is also supported by the distribution of proteolytic activity obtained in the slime fractions (III).

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